Veterinary Immunology and Immunopathology 127 (2009) 106–113

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Somatic hypermutations and isotype restricted exceptionally long CDR3H contribute to antibody diversification in cattle

Azad K. Kaushik [a,*], Marcus E. Kehrli Jr. [b], A. Kurtz [a], S. Ng [a], M. Koti [a], F. Shojaei [a], Surinder S. Saini [a]

[a] Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada
[b] Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center-USDA-Agricultural Research Service, 2300 Dayton Avenue, Ames, IA 50010, USA

ARTICLE INFO

Article history:
Received 30 July 2008
Received in revised form 10 September 2008
Accepted 29 September 2008

Keywords:
Antibody
Cattle
Immunoglobulin
VDJ
Somatic mutations
CDR3H
Bovine leukocyte adhesion deficiency

ABSTRACT

Antibody diversification in IgM and IgG antibodies was analyzed in an 18-month old bovine (*Bos taurus*) suffering from naturally occurring chronic and recurrent infections due to bovine leukocyte adhesion deficiency (BLAD). The BLAD, involving impaired leukocyte β2 integrin expression on leukocytes, develops due to a single point mutation in conserved region of the CD18 gene resulting in substitution of aspartic acid128 with glycine (D128G). Twenty four VDJCμ and 25 VDJCγ recombinations from randomly constructed cDNA libraries, originating from peripheral blood lymphocytes, were examined for the variable-region structural characteristics in IgM and IgG antibody isotypes. These analyses led to conclude that: (a) expression of exceptionally long CDR3H is isotype restricted to cattle IgM antibody; (b) VDJ recombinations encoding IgM with exceptionally long CDR3H undergo clonal selection and affinity maturation via somatic mutations similar to conventional antibodies; (c) somatic mutations contribute significantly to both IgM and IgG antibody diversification but significant differences exist in the patterns of 'hot spot' in the FR1, FR3 and CDR1H and, also, position-dependant amino acid diversity; and (d) transition nucleotide substitutions predominate over transversions in both VDJCμ and VDJCγ recombinations consistent with the evolutionary conservation of somatic mutation machinery. Overall, these studies suggest that both somatic mutations and exceptional CDR3H size generation contribute to IgM and IgG antibody diversification in cattle during the development of immune response to naturally occurring chronic and multiple microbial infections.

Published by Elsevier B.V.

1. Introduction

The immunoglobulins (Ig) are B cell antigen receptors (BCR) and constitute the effectors of adaptive humoral host defences, including those against infectious agents, for which generation of extensive receptor diversity is essential. However, variable-region diversification, both for heavy- (H) and light- (L) chain of an antibody, varies significantly across jawed vertebrates (Butler, 1998; Davis, 2004; Maizels, 2005; Market and Papavasiliou, 2003; Saini and Kaushik, 2003) depending upon relative contribution of germline based combinatorial diversity and post-recombinational mechanisms, e.g., somatic hypermutations and gene conversion. The relative role of H- and L-chains in antigen recognition also varies considerably depending upon antigen specificity or the species involved.

In mice and humans, diverse variable (V) germline genes in juxtaposition with diversity (D) and joining (J)

* Corresponding author at: Department of Molecular and Cellular Biology, Room SCIE 4248, University of Guelph, Guelph, Ontario N1G 2W1, Canada Tel.: +1 519 824 4120.
E-mail address: akaushik@uoguelph.ca (A.K. Kaushik).

0165-2427/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetimm.2008.09.024

genes produce significant diversity, via combinatorial V(D)J recombinations, of the variable-region of heavy chains in the bone marrow throughout life (Kirkham and Schroeder, 1994; Schatz, 2004). Further, most diversity resulting from the amino acid composition is concentrated in the third complementarity-determining region (CDR) of the heavy-chain (CDR3H) that is located in the center of the epitope-binding site. The species with limited germline diversity at the variable-region locus of both the heavy- (V_H) and light- ($V_{\kappa/\lambda}$) chains, such as, chicken (Reynaud et al., 1987), rabbit (Mage, 1998), swine (Butler and Wertz, 2006), sheep (Reynaud et al., 1995) and cattle (Kaushik et al., 2002; Zhao et al., 2006), utilize different post-recombination diversification strategies of the variable region of the antibody heavy-chain since it is mainly involved in antigen recognition. While the single functional V_H and V_λ gene present in chickens is diversified via gene conversion in the bursa of Fabricius (Reynaud et al., 1987), both gene conversion and somatic hypermutations in the appendix contribute to the diversification process in rabbits (Mage, 1998). Select members of gut microflora are also suggested to drive the gut-associated lymphoid tissue (GALT) development in rabbits (Lanning et al., 2000). In swine, extensive junctional diversity provides the major source of diversification during preimmune repertoire development (Butler and Wertz, 2006). Yet a non-antigen dependent somatic hypermutation process during pre-immune repertoire development generates antibody diversity in sheep (Reynaud et al., 1995). A single polymorphic V_H gene family, $BOVV_H1$, is identified in cattle (*Bos taurus*) that is predominantly expressed in the primary antibody repertoire (Berens et al., 1997; Saini et al., 1997; Sinclair et al., 1997). Three V_λ gene families, $V_\lambda1$, $V_\lambda2$ and $V_\lambda3$, are known in cattle (Saini et al., 2003a) where $V_\lambda1a$ and $V_\lambda1b$ genes are mostly expressed in bovine antibodies (Parng et al., 1996; Sinclair et al., 1995). Interestingly, the antibody diversity in cattle also originates from the generation of exceptionally long CDR3H regions (Saini et al., 1999). These unusually long CDR3H regions can extend up to 61 residues in size and often contain multiple even numbered Cys residues. Although multiple Cys residues have been observed in antibody CDR3H of other vertebrates like sharks, camels, dolphins, monotremes (platypus), and occasionally in humans, these Cys-rich CDR3H do not attain the massive size seen in cattle antibodies (Ramsland et al., 2001). The exceptionally long CDR3H regions in cattle antibodies may originate from those germline D_H genes that are unusually long as compared to other species (Shojaei et al., 2003). A unique characteristic of bovine antibodies with exceptionally long CDR3H is that these specifically pair with $V_\lambda1x$, $V_\lambda1d$ and $V_\lambda1e$ genes (Saini et al., 2003a) with conserved Ser90 and Ala91 in the CDR3L regions. The role of the λ-light chains in cattle antibodies is essentially to act as a supporting platform for the extremely long CDR3H regions which are dominantly involved in binding to an antigen (Saini et al., 2003a).

Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive genetic disease (Gerardi, 1996; Kehrli et al., 1992; Nagahata, 2004) of cattle resulting from impaired β2 integrins expression on leukocytes due to a single point mutation in conserved region of the CD18 gene resulting in substitution of aspartic acid128 with glycine (D128G). An additional silent mutation involving replacement of cytosine to thymine at position 775 of CD18 gene is also noted (Shuster et al., 1992). The integrins are heterodimeric proteins consisting of various α chains (CD11a, b, c) non-covalently pairing with the common β chain (CD18) that function as adhesion molecules. Thus, the primary immunodeficiency is characterized with peripheral lymphadenopathy, neutrophilia, hypoalbunemia and hypergammaglobulinemia. The inhibition of diapedesis in the inflammatory response prevents development of normal immune responses to invading pathogens leading to chronic and recurrent infections in BLAD afflicted cattle. The antigen-specific immune responses to tetanus toxoid and rabies virus in BLAD cattle are known to be delayed and impaired (Nagahata et al., 1994). For these reasons, BLAD afflicted cattle provide an immunodeficient model to examine the degree of somatic diversification in IgM and IgG antibody isotypes. The experiments outlined here involved structural analysis of IgM and IgG antibodies from a single BLAD afflicted cattle where these arose as a result of selective pressures on the responding antibody repertoire due to natural infections. These studies demonstrate that cattle IgM and IgG antibodies bear distinct structural properties with regard to exceptional CDR3H size and patterns of somatic hypermutations in the variable-regions during the development of immune response to natural chronic infections.

2. Materials and methods

2.1. Animal

An approximately 18-month-old Holstein heifer afflicted with BLAD was used to obtain lymphocytes. This calf was typical in its clinical presentation (recurrent respiratory disease and diarrhea, a persistent progressive neutrophilia, stunted growth, periodontitis) and was homozygous for the D128G CD18 allele. Peripheral blood was collected into 2× acid citrate dextrose (10%, v/v) by jugular venipuncture. Anticoagulated blood was centrifuged and the plasma layer was discarded. Mononuclear cells from blood were enriched from whole blood buffy coats by buoyant density centrifugation for 40 min at 450 × *g* over a colloidal polyvinylpyrolidone-coated silica gradient (sp gr 1.084). Lymphocyte-enriched cells were harvested and washed once in physiological saline solution and resuspended in Trizol (Gibco BRL, Gaithersburg, MD). The mRNA obtained from lymphocytes was used for construction of two cDNA libraries.

2.2. Polymerase chain reaction (PCR)

Single strand cDNA was synthesized from 7 μg of total RNA, isolated by Trizol reagent (Gibco-BRL, Burlington, Ontario), using polydT primer (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec). All VDJ recombinations were PCR amplified (Saini et al., 1997) in an unbiased manner using high fidelity HF Expand system (Boehringer-

Mannheim Canada, Laval, Quebec) and the following specific primers:

VDJCμ: 5′ primer (5′AGCTCGAGATGAACCCACTGTG 3′) from the leader sequence and 3′ primer from Cμl exon (5′AGACTAGTGAAGACTCTCGGGTGTG3′).
VDJCγ: 5′ primer (5′AGCTCGAGATGAACCCACTGTG 3′) from the leader sequence and 3′ primer from Cγ1 exon (5′ AGACTAGTGGCTGTGGTGGAGGC 3′).

These primers, corresponding to conserved leader and constant (Cμ/Cγ) region sequence, led to unbiased random amplification of all V_H genes expressed in cattle (Saini et al., 1997; Sinclair et al., 1997). Various PCR steps included a hot start (95 °C for 2 min), denaturation at 95 °C for 1 min, annealing at 65 °C (VDJCγ) or 68 °C (VDJCμ) for 1 min and extension at 72 °C for 1 or 2 min up to a total of 30 cycles. A final elongation step of 72 °C for 7 min was included at the end of the final cycle. The reaction conditions included 1.5 mM $MgCl_2$ concentration, 0.8 μM of each primer and 2.5 U of Taq polymerase (PerkinElmer, Woodbridge, Ontario).

2.3. cDNA Library construction

Two unbiased random cDNA libraries, BLADM (VDJCμ) and BLADG (VDJCγ) were constructed from RNA isolated from peripheral blood lymphocytes of an 18-month-old Holstein calf afflicted with BLAD, as described (Cassady-Cain and Kaushik, 2006). Briefly, purified PCR amplified products (Qiaquick; Qiagen, Mississauga, Ontario) were blunt ended using T4 DNA polymerase (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec) and gel purified (Qiaex II, Qiagen, Mississauga, Ontario) prior to random ligation into the ZeroBlunt vector (Invitrogen Canada, Burlington, Ontario). The ligate was used to transform TOP 10 competent *E. coli* using heat shock (Cassady-Cain and Kaushik, 2006). Recombinant plasmids isolated from bacterial colonies were tested for the presence of an insert by digestion with EcoR1 followed by Southern blot hybridization with bovine V_H gene specific DNA probe as described (Saini et al., 1997). The DNA sequence of various VDJ recombinations in each of the libraries, in both directions, was obtained using M13 reverse and M13 forward primers (MOBIX, McMaster University, Hamilton).

2.4. DNA sequence and protein analysis

The unique VDJ recombination sequences, 24 VDJCμ (GenBank accession numbers: DQ487734–DQ487757) and 25 VDJCγ (GenBank accession numbers: DQ485764–DQ485788), excluding duplicate isolates were numbered according to Kabat (Johnson and Wu, 2000) and analyzed using DNA Star (DNAStar Inc., Madison Wisconsin) and BLAST programs (Altschul et al., 1990). The strategy used for hot spot analysis (AGPy:TCN) exploited the fact that nucleotide substitutions resulting from somatic hypermuation are not distributed randomly throughout the V_H gene sequence but favor targeting to individual hot spots (Betz et al., 1993; Rogozin and Kolchanov, 1992; Sharpe et al., 1991). Such a selective and targeted approach was used since all bovine germline genes and allelic differences are not fully characterized. Similarly, analysis for transitions was focused on RGYW motifs (Rogozin et al., 2001) upon comparison with available germline V_H genes. The base usage that may contribute to hypermutation was explored by examining A + T:G + C ratio (Padlan, 1997). The amino acid composition analysis of V_H encoded region for strongly basic (K, R), acidic (D, E), hydrophobic (A, I, L, F, W) and polar (N, C, Q, S, T, Y) was performed using DNA Star program. The GraphPad InStat version 3.05 (GraphPad Software, San Diego, California, USA) was used for calculating Student's *t* test or one way ANOVA to determine statistical significance (p value < 0.05) of various test variables.

3. Results

3.1. Exceptionally long CDR3H occurs only in IgM antibodies of cattle

Analysis of 24 VDJCμ and 25 VDJCγ recombinations and deduced amino acids obtained (Supplementary Figs. 1 and 2) from unbiased random cDNA library from a single calf afflicted with BLAD and associated chronic infections revealed an average CDR3H size of 21.7 ± 1.8 and 18.2 ± 1.3 codons in IgM and IgG antibodies, respectively (Table 1). However, the CDR3H size varied considerably between IgM (13–54 codons) and IgG antibodies (2–26 codons). None of the IgG antibodies showed the exceptionally long CDR3H >30 amino acids. These observations are consistent with an average CDR3H size of 22 ± 11 amino acids, ranging between 3 and 61 codons, of random 102 cattle IgM antibodies from non-BLAD affected apparently healthy cattle (Saini et al., 1999, 1997; Saini and Kaushik, 2002). None of the IgG antibodies of cattle analyzed here, including those from two other groups (Berens et al., 1997; Sinclair et al., 1997), express an exceptionally long CDR3H observed in IgM antibodies (>30 ≤ 61 amino acids). Therefore, characteristically long CDR3H is a feature confined to IgM antibody isotype in cattle. In addition, such an exceptional CDR3H size is outside the range noted in IgM and IgG antibodies from other ruminant species such as sheep (3–23), camel (10–24) and llama (5–26). Thus, a subset of cattle IgM antibodies is structurally distinct with regard to CDR3H size, a principal determinant of antigen specificity, as compared to IgG antibodies.

The predominant expression of germline J_H1 gene in both VDJCμ and VDJCγ recombinations encoding varying

Table 1
Structural characteristics of the variable regions of IgM and IgG antibodies from single cattle afflicted with bovine leukocyte adhesion deficiency.

Characteristics	BLADM (n = 24)	BLADG (n = 25)
Total amino acids (a.a.)	130 ± 9[a] (121–153)	126 ± 7 (110–134)
CDR3H Size	21.7 ± 1.8 (13–54)	18.2 ± 1.3 (2–26)
Strongly Basic a.a. (K, R)	10.4 ± 1.6 (8–12)	9.8 ± 1.3 (8–12)
Strongly Acidic a.a. (D, E)	9.8 ± 2.0 (7–15)	8.6 ± 1.5 (6–12)
Hydrophobic a.a. (A, I, L, F, W)	39.3 ± 2.8 (34–46)	39 ± 3 (32–45)
Polar a.a. (N, C, Q, S, T, Y)	52 ± 5 (45–68)	51 ± 4 (43–58)
Isoelectric point	7.2 ± 1.2 (4.6–8.5)	7.4 ± 1.4 (4.8–9.2)

[a] Mean ± standard error; parentheses indicate range.

Table 2
Mutational hot spots and (A + T):(G + C) ratios in bovine VDJCμ and VDJCγ recombinations.

Region	AGPY:TCN ratio[a] (mean ± SE)			A + T:G + C ratio (mean ± SE)		
	BLADM (VDJCμ) clones (n = 24)	BLADG (VDJCγ) clones (n = 25)	p-Value[b]	BLADM (VDJCμ) clones (n = 24)	BLADG (VDJCγ) clones (n = 25)	p-Value[b]
FR1	**0.43 ± 0.03**	**0.36 ± 0.02**	0.049	0.58 ± 0.008	0.58 ± 0.006	>0.99
FR2	0.042 ± 0.04	0	–	0.47 ± 0.016	0.46 ± 0.014	0.643
FR3	**1.3 ± 0.15**	**1.8 ± 0.18**	0.04	**0.92 ± 0.002**	**0.88 ± 0.016**	0.019
CDR1	**1.4 ± 0.14**	**0.7 ± 0.12**	0.0005	1.3 ± 0.084	1.1 ± 0.06	0.057
CDR2	1.1 ± 0.16	0.8 ± 0.14	0.187	**1.21 ± 0.047**	**1.13 ± 0.046**	0.022
CDR3	1.1 ± 0.25	1.2 ± 0.24	0.771	1.6 ± 0.10	1.5 ± 0.12	0.530

[a] Py: pyrimidine (C or T); N: A/T/C/G.
[b] Two-tailed unpaired '*t*' test; significant statistical differences are shown in bold.

CDR3H size (Supplementary Fig. 3) suggests that it does not play a significant role in the origin of unusually long CDR3H. This is consistent with the suggestion that D-gene element use is the single most important determinant of CDR3 length distribution in all immune receptor chains (Rock et al., 1994). Thus, it is likely that unusually long CDR3H in IgM antibodies are directly encoded by long germline D-gene elements (Shojaei et al., 2003) without any role of J_H genes. Overall, these observations suggest that the exceptional CDR3H size (≥30 amino acid) is restricted to the IgM isotype of cattle antibodies, though bovine IgMs, in general, express a relatively long CDR3H as compared to IgG antibodies.

3.2. Somatic mutation hot spots differ in VDJCμ and VDJCγ recombinations expressed in bovine B cells

Analysis of AGPy:TCN ratio in framework regions (FRs) and CDRs of VDJCμ and VDJCγ recombinations showed statistically significant differences in the occurrence of mutational hot spots in the FR1 and CDR1 regions ($p < 0.05$) of IgM and IgG antibodies (Table 2). Obviously, such differences result from accumulation of mutations in the FR1 and CDR1 regions of IgG leading to loss of mutational hot spots in contrast to IgM antibodies. Both IgM and IgG antibodies, however, had comparable and statistically insignificant ($p > 0.05$) mutational hot spots in CDR2H region as these may have undergone extensive mutations. Surprisingly, statistically significant differences ($p < 0.05$) in mutational hot spots were noted in the FR3 region of IgM (1.3 ± 0.8) and IgG (1.8 ± 0.9) antibody isotypes. A relatively high AGPy:TCN ratio in the FR3 of VDJCγ recombinations suggests *de novo* generation of mutational hot spots as an outcome of somatic mutations during class switch recombination. By contrast, the FR2 region of both IgM and IgG antibodies had a minimal incidence of mutational hot spots (Table 2) and, is, thus, most conserved amongst the V_H encoded FRs. One way ANOVA showed significant differences ($p < 0.0001$) in mutational hot spot incidence in both VDJCμ and VDJCγ recombinations. Further, FR3 region in both groups *per se* had significantly higher mutational hot spots as compared to FR1 (VDJCμ—$p < 0.01$; VDJCγ—$p < 0.001$) and FR2 (VDJCμ—$p < 0.001$; VDJCγ—$p < 0.001$) regions. No unequivocal evidence for gene conversion was noted in VDJCμ and VDJCγ recombinations in cattle antibodies (data not shown) based on comparison with the available germilne V_H genes (Table 3). Significant differences ($p < 0.05$) in A + T:G + C ratio of FR3 and CDR2H were observed in VDJCμ and VDJCγ recombinations (Table 2). The FR3 region *per se* significantly differed in A + T:G + C ratio from FR1 (VDJCμ—$p < 0.01$; VDJCγ—$p < 0.05$) and FR2 (VDJCμ—$p < 0.001$; VDJCγ—$p < 0.001$) regions of both VDJCμ and VDJCγ recombinations. Taken together, these observations demonstrate significant differences in the patterns of somatic mutations between IgM and IgG antibody isotypes of cattle. This is particularly significant that these differences are evident in the immune repertoire of a single animal where B cells underwent selection pressures from natural infections.

Although somatic mutations occur both in IgM and IgG antibodies, position-dependant differences in amino acid diversity (Fig. 1) are significant between the two isotypes ($p < 0.0001$). The amino acid substitutions in IgM and IgG antibodies at positions 52 ($p < 0.01$), 53 ($p < 0.01$) and 54 ($p < 0.001$) of the V_H encoded CDR2H were remarkable for most diversification. These observations suggest that both IgM and IgG antibody repertoires in cattle are diversified via somatic mutations but significant differences in the patterns of mutational hot spots (FR1, FR3 and CDR1H) and position-dependant diverse amino acid substitutions in the CDR2H exist between IgM and IgG isotypes under selection pressures from natural infections.

Table 3
Percent similarity among available bovine germline V_H genes[a].

Group	Germline gene	Members (% homology)	Homology range
1	BF1H1	BF4E9 (100%) gl. 110.20 (99.1%)	99.1–100%
2	BF2D12	U55168 (95.4%) U55170 (96.8%)]U55171 (96.8%) U55172 (99.3%) U55173 (99.3%) U55174 (97.5%)	95.4–99.3%
3	BF3A11	BF2B5 (98.3%) BF5F10 (99.7%) U55169 (95.8%) U55175 (95.8%)	95.8–99.7%
4	BF3H11	U55170 (95.1%) U55171 (95.1%)	95.1%
5	U55166	U55167 (98.9%)	98.9%

[a] Available via GenBank.

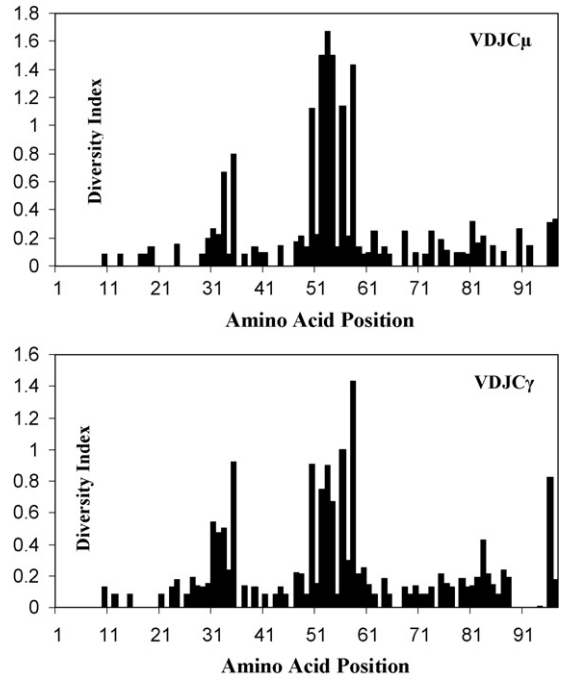

Fig. 1. Diversity indices of 24 VDJCμ (a) and 25 VDJCγ (b) recombinations from 18-month-old cow afflicted with BLAD. Diversity indices were determined as: Number of different amino acids at the position/Number of most frequent amino acid at the position.

3.3. Transitions predominate over transversions in both VDJCμ and VDJCγ recombinations

Since all bovine germline genes are not yet characterized, we analyzed nucleotide substitutions by first grouping available bovine germline genes on the basis of >95% sequence similarity (Table 3) followed by identification of conserved RGYW motifs, known targets for mutations (Diaz et al., 2001; Rogozin et al., 2001) among all bovine germline V_H genes (Table 4). The nucleotide substitutions at these motifs showed predominant occurrence of transitions (73%) over transversions (27%) in both VDJCμ and VDJCγ recombinations (Table 5) and such a pattern of mutations in purines is consistent with the strand polarity (Neuberger et al., 1998). The G → A transition (VDJCμ—43.8%; VDJCγ—38.4%) and G → C transversion (VDJCμ—19.6%; VDJCγ—14.5%) substitutions were the most frequent. The motif TAA was noted to be conserved only at position 177–179 of CDR2H of both VDJCμ and VDJCγ recombinations that led to few transition and transversion substitutions. These observations, similar to other species, suggest a predominant occurrence of transition nucleotide substitutions in bovine immunoglobulins consistent with a role for strand polarity and evolutionary conservation of somatic hypermutation machinery.

Table 4
Conserved RGYW motifs in available bovine germline[a] V_H genes.

Region	Position	Motif
FR1	8–11	AGCT
CDR1	88–91	AGCA
	91–94	AGCT/A
	103–106	GGCT
FR2	117–120	GGC/TT
	140–143	GGC/TT
CDR2	148–151	GGTA
	154–157	AGTA
	166–169	AGCA
	172–175	A/GGCT
FR3	197–200	GGCT
	202–205	AGCA
	247–250	AGCA

[a] Germline V_H gene grouping in Table 3.

Table 5
Percent nucleotide substitutions in bovine IgM and IgG antibodies.

	To: G	A	T	C	Total	
From:						
VDJCμ						
G	–	43.8	11.6	19.6	75	83
A	7.1	–	0.9	0	8.0	
T	0.9	0	–	1.8	2.7	17
C	2.7	2.7	8.9	–	14.3	
VDJCγ						
G	–	38.4	10.9	14.5	63.8	81
A	15.2	–	0.7	1.5	17.4	
T	2.9	0	–	1.5	4.4	19
C	4.4	2.9	7.3	–	14.5	
	VDJCμ		VDJCγ		VDJCμ + VDJCγ	
Transitions	82/112(73.21%)		101/138(73.19%)		73.2%	
Transversions	30/112(26.79%)		37/138(26.81%)		26.8%	

3.4. Somatic mutations diversify VDJ recombinations including those encoding exceptionally long CDR3H

Although the pattern and degree of diversification varied between IgM and IgG antibodies in cattle with BLAD primary immunodeficiency, these data showed extensive diversity in the CDRHs, and to some extent in FR3 in contrast to FR1 and FR2 (Fig. 1) underscoring the importance of somatic nucleotide substitution mutations in cattle antibody diversification. The IgMs with exceptionally long CDR3H were expressed at approximately 8% in BLAD afflicted cattle with recurrent and chronic infection, similar to those noted in peripheral B cells from adult cattle (Saini et al., 1999). Nevertheless, occurrence of somatic mutations in the V_H encoded CDR1H region, e.g., clone Bladm20 (Fig. 2) suggests that the VDJ recombinations encoding exceptionally long CDR3H undergo affinity maturation in the periphery similar to classical antibodies during the development of immune response to exogenous antigens. This is consistent with the fact that somatic mutations were also noted in the CDR2H and FR3 of VDJ recombinations encoding exceptionally long CDR3H in non-BLAD cattle (Saini et al., 1999). Inspection of VDJ recombinations from BLAD afflicted cattle revealed somatic mutations in the CDR1H of IgM with exceptionally long CDR3H as well, for example, Bladm20 clone (Fig. 2), where valine was substituted with isoleucine due to substitution mutation. Thus, VDJ recombinations encoding

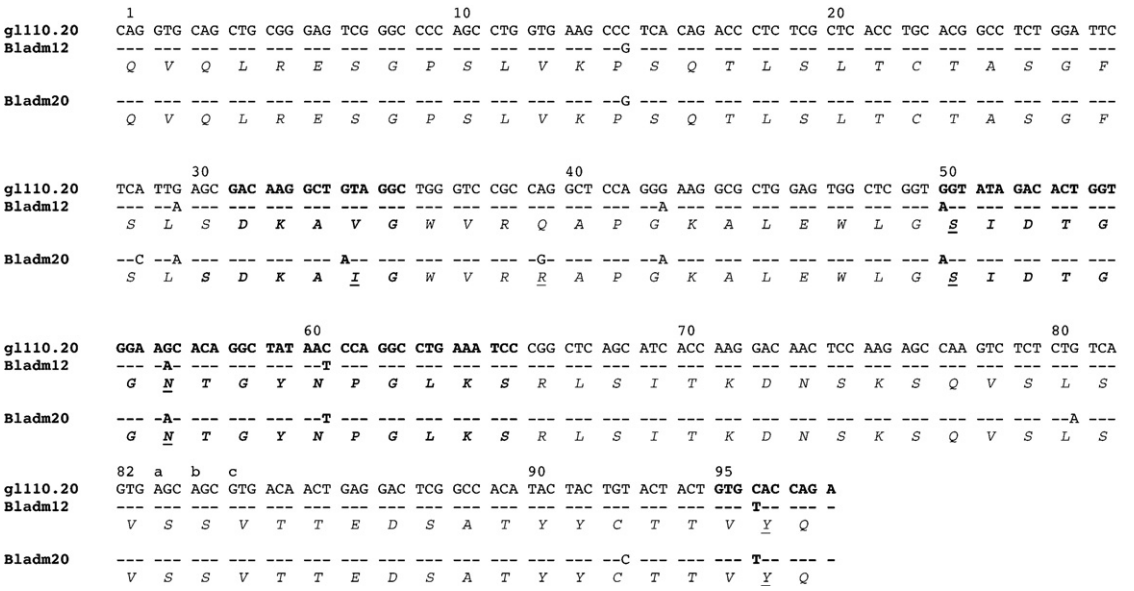

Fig. 2. Comparison of VDJ recombinations (Bladm12 and Bladm20) encoding exceptionally long CDR3H with the corresponding germline $V_H$gene (gl1 10.20). Note amino acid replacement in CDR1H, valine to isoleucine, of Bladm20 clone originating from single nucleotide substitution somatic mutation.

exceptionally long CDR3H undergo somatic mutations in the V_H encoded CDRHs, FR2 and FR3 during affinity maturation of immune response to foreign antigens. The variable-region structural properties of IgM and IgG antibodies (Table 1) reveals remarkable size variations in IgM and IgG that are essentially due to CDR3H size differences. However, no significant differences in amino acids that constitute the variable-region of either IgM or IgG antibodies are noted. Similar to classical antibodies, IgM antibodies with unusually long CDR3H undergo clonal selection and affinity maturation via somatic mutations, although class switch recombination precludes retention of such a CDR3H in IgG antibodies for reasons not yet understood.

4. Discussion

While antibody diversification, a constituent of adaptive humoral responses, is conserved across vertebrates, significant differences across species emerge with regard to diversification mechanisms depending upon germline sequence divergence required for combinatorial diversity. Earlier, we reported that cattle, a species with limited potential germline encoded combinatorial diversity, employ extensive CDR3H size heterogeneity (Saini and Kaushik, 2002) together with generation of exceptionally long CDR3H for diversifying the antibody repertoire (Saini et al., 1999). The experiments outlined here examined the degree and pattern of diversification in the variable-region of IgM and IgG antibody isotypes from 18-month-old cattle afflicted with BLAD since this primary immunodeficiency results in naturally occurring chronic and recurrent infections leading to extensive mutations in the antibody variable-region. These studies led us to conclude that distinct variable-region structural properties characterize IgM and IgG isotypes of cattle antibodies under selective pressures from natural infections: (a) the exceptionally long CDR3H is restricted to the IgM isotype; (b) VDJ recombinations encoding IgM with exceptionally long CDR3H undergo clonal selection and affinity maturation via somatic mutations similar to conventional antibodies; and (c) although somatic hypermutations occur both in IgM and IgG, mutational hot spots (FR1, FR3 and CDR1H regions) and A + T:G + C ratios (FR3 and CDR2H) differ among these isotypes. Similar to other species (Neuberger et al., 1998), transitions predominate over transversions in both IgM and IgG antibodies of cattle. The exceptionally long CDR3H is not observed in IgG antibodies originating from BLAD afflicted cattle, consistent with other studies (Berens et al., 1997; Kaushik et al., 2002; Saini et al., 1999, 1997; Saini and Kaushik, 2002; Sinclair et al., 1997). Nevertheless, cattle IgG antibodies tend to have a relatively long CDR3H as compared to other species such as sheep (Reynaud et al., 1995), camels (De Genst et al., 2006) and humans (Kirkham and Schroeder, 1994; Schatz, 2004). It is likely that exclusive expression of remarkably long CDR3H ($>30 \leq 61$ amino acid) in IgM antibodies is due to specific structural requirements (Saini et al., 1999, 2003a) with regard to specific gl.1 10.20 germline V_H gene utilization and restricted $V_H + V_\lambda$ pairing. The role of D-gene segments is yet to be fully determined since the longest D-gene isolated to date is potentially capable of encoding 49 codons and does not yet explain the origin of CDR3H as long as 61 amino acids. The IgM antibodies with exceptionally long CDR3H also undergo clonal selection and affinity maturation via somatic mutations similar to conventional antibodies. While extensive somatic hypermutations, a phenomenon associated with class switch recombination, are noted in both IgM and IgG antibodies, it is not clear why the variable-region comprising exceptionally long CDR3H is not retained in IgGs subsequent to class switch recombination. One possibility could be that B

cells expressing IgM with exceptionally long CDR3H as BCR on cell surface do not undergo class switch recombination, similar to murine peritoneal B-cells known to express only IgM and IgG3 isotypes (Kaushik et al., 1988), but this needs to be experimentally tested. Alternatively, the γ-constant domain is unable to sustain a variable-region with exceptional long CDR3H due to structural constraints and B cells expressing such a BCR are negatively selected during the development of an immune response and undergo apoptosis in the germinal center. Nevertheless, consistent expression of IgM with exceptionally long CDR3H at 8% level in the periphery suggests that these are stably expressed during the development of immune response to naturally occurring recurrent infections, although some of these are known to encode polyspecific reactivity (Saini et al., 2003b). Since IgM antibodies with exceptionally long CDR3H undergo clonal selection and affinity maturation similar to IgG, as evidenced by somatic mutations in the CDRHs, these contribute to humoral host defence in the event of infection. Such IgM antibodies are capable of providing increased configurational diversity via exceptionally long CDR3H (Ramsland et al., 2001) as well as single point mutations resulting in amino acid substitutions.

Since transitions predominate over transversions of single nucleotide substitutions in cattle IgM and IgG antibodies similar to other species such as shark, frogs, camels, pig, sheep and humans (Diaz et al., 2001; Green et al., 1998; Neuberger et al., 2005), it is likely that the somatic machinery involved is evolutionarily conserved. It needs to be determined whether in cattle, similar to sheep (Reynaud et al., 1995), somatic single nucleotide mutations contribute to non-antigen dependant diversification of the preimmune immunoglobulin repertoire, apart from generating high affinity antibodies in the periphery. The experiments outlined here confirm that somatic mutations and CDR3H size of cattle antibodies, rather than gene conversion, contribute to diversification of the variable-region of the heavy-chain mainly involved in antigen recognition. Nevertheless, gene conversion has been noted to be involved in λ-light chain diversification in cattle where pseudogenes acted as donor (Parng et al., 1996). The process of somatic mutations (Neuberger et al., 2005), involving initiation by the enzyme Activation Induced Deaminase (AID) where it deamidates cytidine residues to uracil (Pham et al., 2003) followed by excision of uracil and repair by certain error-prone polymerases (Green et al., 1998), is known to favour transitions over transversions at a 3:1 ratio (Betz et al., 1993). Similar to these observations, the mutated antibodies of cattle analyzed in this study show a ratio of 2.7:1. Further, the experiments outlined here demonstrate that conserved RGYW motif is also a 'hot spot' and a target of somatic mutations in cattle antibodies. Since all bovine germline genes are not yet sequenced, the possibility of allelic variation was excluded by focusing analysis on the RGYW motif conserved in all the available bovine germline V_H genes. The *de novo* generation of hot spots as a result of single nucleotide substitutions observed in IgG antibodies is supported by similar observations in cultured B cells (Lin et al., 1997) and light-chain transgene suggesting involvement of higher order structure (Goyenechea and Milstein, 1996) or more distant sequences (Lin et al., 1997). In addition to RGYW, WA motif is also known as an optimal descriptor of somatic hypermutation but its frequency was observed to be relatively low in the variable-region of cattle antibodies.

Although specific immune responses, including generation of antigen-specific IgG, to tetanus toxoid and rabies virus in BLAD cattle are suggested to be delayed and impaired (Shuster et al., 1992), it is obvious that this is not due to inherent lack of antibody diversification since mutation machinery is functional. Such a delay is most likely due to less efficient interactions between antigen-presenting cells and T lymphocytes due to defective β2-integrins as evidenced from graft rejection in cattle afflicted with BLAD (Muller et al., 1995). The frequency of B cells specific to an epitope present on an infectious agent is suggested to be $1–10 \times 10^{-5}$ (Bachmann and Zinkernagel, 1997) without taking into consideration the newly emerging microbial variants during the infectious process (Ohlin and Zouali, 2003). Given the limited germline combinatorial diversity for the variable region of the heavy-chain, somatic single point mutations constitute the major mechanism for diversifying antibody response to the emerging polymorphic microbial epitopes as a result of infection in cattle. This is especially important in cattle as the variable-region of the heavy-chain is mainly involved in antigen recognition where λ-light chains provide only structural support (Saini et al., 2003a).

Acknowledgements

The authors would like to gratefully acknowledge the review of the manuscript by Dr. James A. Harp, National Animal Disease Center-USDA-Agricultural Research Service, Ames, Iowa. The research was supported by operating grants from NSERC Canada, Ontario Cattlemen's Association and OMAF research program, in addition to a grant from Food System Biotechnology Centre, University of Guelph to Dr. Azad K. Kaushik.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2008.09.024.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Bachmann, M.F., Zinkernagel, R.M., 1997. Neutralizing antiviral B cell responses. Annu. Rev. Immunol. 15, 235–270.
Berens, S.J., Wylie, D.E., Lopez, O.J., 1997. Use of a single VH family and long CDR3s in the variable region of cattle Ig heavy chains. Int. Immunol. 9, 189–199.
Betz, A.G., Rada, C., Pannell, R., Milstein, C., Neuberger, M.S., 1993. Passenger transgenes reveal intrinsic specificity of the antibody hypermutation mechanism: clustering, polarity, and specific hot spots. Proc. Natl. Acad. Sci. U.S.A. 90, 2385–2388.
Butler, J.E., 1998. Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals. Rev. Sci. Tech. 17, 43–70.

Butler, J.E., Wertz, N., 2006. Antibody repertoire development in fetal and neonatal piglets. XVII. IgG subclass transcription revisited with emphasis on new IgG3. J. Immunol. 177, 5480–5489.
Cassady-Cain, R.L., Kaushik, A.K., 2006. Increased negative selection impairs neonatal B cell repertoire but does not directly lead to generation of disease-associated IgM auto-antibodies. Int. Immunol. 18, 661–669.
Davis, M.M., 2004. The evolutionary and structural 'logic' of antigen receptor diversity. Semin. Immunol. 16, 239–243.
De Genst, E., Saerens, D., Muyldermans, S., Conrath, K., 2006. Antibody repertoire development in camelids. Dev. Comp. Immunol. 30, 187–198.
Diaz, M., Flajnik, M.F., Klinman, N., 2001. Evolution and the molecular basis of somatic hypermutation of antigen receptor genes. Philos. Trans. R. Soc. Lond. B: Biol. Sci. 356, 67–72.
Gerardi, A.S., 1996. Bovine leucocyte adhesion deficiency: a review of a modern disease and its implications. Res. Vet. Sci. 61, 183–186.
Goyenechea, B., Milstein, C., 1996. Modifying the sequence of an immunoglobulin V-gene alters the resulting pattern of hypermutation. Proc. Natl. Acad. Sci, U.S.A. 93, 13979–13984.
Green, N.S., Lin, M.M., Scharff, M.D., 1998. Somatic hypermutation of antibody genes: a hot spot warms up. Bioessays 20, 227–234.
Johnson, G., Wu, T.T., 2000. Kabat database and its applications: 30 years after the first variability plot. Nucleic Acids Res. 28, 214–218.
Kaushik, A., Lim, A., Poncet, P., Ge, X.R., Dighiero, G., 1988. Comparative analysis of natural antibody specificities among hybridomas originating from spleen and peritoneal cavity of adult NZB and BALB/c mice. Scand. J. Immunol. 27, 461–471.
Kaushik, A., Shojaei, F., Saini, S.S., 2002. Novel insight into antibody diversification from cattle. Vet. Immunol. Immunopathol. 87, 347–350.
Kehrli Jr., M.E., Ackermann, M.R., Shuster, D.E., van derMaaten, M.J., Schmalstieg, F.C., Anderson, D.C., Hughes, B.J., 1992. Bovine leukocyte adhesion deficiency. Beta 2 integrin deficiency in young Holstein cattle. Am. J. Pathol. 140, 1489–1492.
Kirkham, P.M., Schroeder Jr., H.W., 1994. Antibody structure and the evolution of immunoglobulin V gene segments. Semin. Immunol. 6, 347–360.
Lanning, D., Sethupathi, P., Rhee, K.J., Zhai, S.K., Knight, K.L., 2000. Intestinal microflora and diversification of the rabbit antibody repertoire. J. Immunol. 165, 2012–2019.
Lin, M.M., Zhu, M., Scharff, M.D., 1997. Sequence dependent hypermutation of the immunoglobulin heavy chain in cultured B cells. Proc. Natl. Acad. Sci. U.S.A. 94, 5284–5289.
Mage, R.G., 1998. Diversification of rabbit VH genes by gene-conversion-like and hypermutation mechanisms. Immunol. Rev. 162, 49–54.
Maizels, N., 2005. Immunoglobulin gene diversification. Annu. Rev. Genet. 39, 23–46.
Market, E., Papavasiliou, F.N., 2003. V(D)J recombination and the evolution of the adaptive immune system. PLoS Biol. 1, E16.
Muller, K.E., Rutten, V.P., Becker, C.K., Hoek, A., Bernadina, W.E., Wentink, G.H., Figdor, C.G., 1995. Allograft rejection in cattle with bovine leukocyte adhesion deficiency. Vet. Immunol. Immunopathol. 48, 55–63.
Nagahata, H., 2004. Bovine leukocyte adhesion deficiency (BLAD): a review. J. Vet. Med. Sci. 66, 1475–1482.
Nagahata, H., Nochi, H., Sanada, Y., Tamoto, K., Noda, H., Kociba, G.J., 1994. Analysis of mononuclear cell functions in Holstein cattle with leukocyte adhesion deficiency. Am. J. Vet. Res. 55, 1101–1106.
Neuberger, M.S., Di Noia, J.M., Beale, R.C., Williams, G.T., Yang, Z., Rada, C., 2005. Somatic hypermutation at A. T pairs: polymerase error versus dUTP incorporation. Nat. Rev. Immunol. 5, 171–178.
Neuberger, M.S., Ehrenstein, M.R., Klix, N., Jolly, C.J., Yelamos, J., Rada, C., Milstein, C., 1998. Monitoring and interpreting the intrinsic features of somatic hypermutation. Immunol. Rev. 162, 107–116.
Ohlin, M., Zouali, M., 2003. The human antibody repertoire to infectious agents: implications for disease pathogenesis. Mol. Immunol. 40, 1–11.
Padlan, E.A., 1997. Does base composition help predispose the complementarity-determining regions of antibodies to hypermutation? Mol. Immunol. 34, 765–770.
Parng, C.L., Hansal, S., Goldsby, R.A., Osborne, B.A., 1996. Gene conversion contributes to Ig light chain diversity in cattle. J. Immunol. 157, 5478–5486.
Pham, P., Bransteitter, R., Petruska, J., Goodman, M.F., 2003. Processive AID-catalysed cytosine deamination on single-stranded DNA simulates somatic hypermutation. Nature 424, 103–107.
Ramsland, P.A., Kaushik, A., Marchalonis, J.J., Edmundson, A.B., 2001. Incorporation of long CDR3s into V domains: implications for the structural evolution of the antibody-combining site. Exp. Clin. Immunogenet. 18, 176–198.
Reynaud, C.A., Anquez, V., Grimal, H., Weill, J.C., 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. Cell 48, 379–388.
Reynaud, C.A., Garcia, C., Hein, W.R., Weill, J.C., 1995. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. Cell 80, 115–125.
Rock, E.P., Sibbald, P.R., Davis, M.M., Chien, Y.H., 1994. CDR3 length in antigen-specific immune receptors. J. Exp. Med. 179, 323–328.
Rogozin, I.B., Kolchanov, N.A., 1992. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. Biochim. Biophys. Acta 1171, 11–18.
Rogozin, I.B., Pavlov, Y.I., Bebenek, K., Matsuda, T., Kunkel, T.A., 2001. Somatic mutation hotspots correlate with DNA polymerase eta error spectrum. Nat. Immunol. 2, 530–536.
Saini, S.S., Allore, B., Jacobs, R.M., Kaushik, A., 1999. Exceptionally long CDR3H region with multiple cysteine residues in functional bovine IgM antibodies. Eur. J. Immunol. 29, 2420–2426.
Saini, S.S., Farrugia, W., Ramsland, P.A., Kaushik, A.K., 2003a. Bovine IgM antibodies with exceptionally long complementarity-determining region 3 of the heavy chain share unique structural properties conferring restricted VH + Vlambda pairings. Int. Immunol. 15, 845–853.
Saini, S.S., Hein, W.R., Kaushik, A., 1997. A single predominantly expressed polymorphic immunoglobulin VH gene family, related to mammalian group, I, clan, II, is identified in cattle. Mol. Immunol. 34, 641–651.
Saini, S.S., Kaushik, A., 2002. Extensive CDR3H length heterogeneity exists in bovine foetal VDJ rearrangements. Scand. J. Immunol. 55, 140–148.
Saini, S.S., Kaushik, A., Basrur, P.K., Yamashiro, S., 2003b. Ultrastructural and immunologic characteristics of mouse × cattle xenogeneic hybridomas originating from bovine leukemia virus-infected cattle. Vet. Pathol. 40, 460–464.
Saini, S.S., Kaushik, A., 2003. Immunoglobulin genes and their diversification in vertebrates. Curr. Trends Immunol. 4, 162–176.
Schatz, D.G., 2004. V(D)J recombination. Immunol. Rev. 200, 5–11.
Sharpe, M.J., Milstein, C., Jarvis, J.M., Neuberger, M.S., 1991. Somatic hypermutation of immunoglobulin kappa may depend on sequences 3′ of C kappa and occurs on passenger transgenes. EMBO J. 10, 2139–2145.
Shojaei, F., Saini, S.S., Kaushik, A.K., 2003. Unusually long germline DH genes contribute to large sized CDR3H in bovine antibodies. Mol. Immunol. 40, 61–67.
Shuster, D.E., Kehrli Jr., M.E., Ackermann, M.R., Gilbert, R.O., 1992. Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. Proc. Natl. Acad. Sci. U.S.A. 89, 9225–9229.
Sinclair, M.C., Gilchrist, J., Aitken, R., 1995. Molecular characterization of bovine V lambda regions. J. Immunol. 155, 3068–3078.
Sinclair, M.C., Gilchrist, J., Aitken, R., 1997. Bovine IgG repertoire is dominated by a single diversified VH gene family. J. Immunol. 159, 3883–3889.
Zhao, Y., Jackson, S.M., Aitken, R., 2006. The bovine antibody repertoire. Dev. Comp. Immunol. 30, 175–186.